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Comparison of Assay of Coliform Enterotoxins by Conventional Techniques Versus In Vivo Intestinal Perfusion

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Thirty-six strains of coliform bacteria were tested for enterotoxigenicity both by conventional assays, including the Y-1 adrenal and Chinese hamster ovary cell assays for heat-labile toxin and the suckling mouse assay for heat-stable toxin, and by determining the ability of graded concentrations of ultrafiltrate high- or low-molecular-weight toxin preparations to induce water secretion during in vivo perfusion in the rat jejunum. The ultrafiltrates of all 18 strains isolated from persons with infectious diarrheal disease, including seven of *Escherichia coli*, seven of *Klebsiella pneumoniae*, and four of *Enterobacter cloacae*, contained one (nine strains) or two (nine strains) potent toxin fractions (resembling either heat-labile or heat-stable toxin in terms of apparent molecular weight and heat lability characteristics) that induced water secretion at perfusion concentrations of 10 ng/ml or less. Unconcentrated broth filtrates of five of the *E. coli* strains and two of *Klebsiella* reacted positively in one or more of the conventional assay systems. Concentrated ultrafiltrates from two strains that were negative in the in vitro assays for heat-labile toxin were tested and also proved to be inactive in these test systems. None of 18 strains isolated from control sources produced, in the ultrafiltrates, enterotoxins capable of inducing water secretion at low concentrations, and none reacted positively in the conventional assays. These results indicate that some strains of coliform bacteria elaborate potent toxin materials that are capable of inducing water secretion and can be detected by perfusion of concentrated ultrafiltrates but not by conventional assay systems for enterotoxigenicity. Whether this represents quantitative or qualitative differences between the toxin materials that stimulate these different test systems remains to be established.

The rather cumbersome technique of testing for the heat-labile (LT) and heat-stable (ST) enterotoxins of *Escherichia coli* with the rabbit ligated ileal loop has been largely replaced by simpler, more rapid techniques. LT is now assayed with in vitro techniques, including tissue culture in Y-1 adrenal (6) or Chinese hamster ovary (CHO) (15) cells, passive immune hemolysis (10), solid-phase radioimmunoassay (4, 14), or enzyme-linked immunoabsorbent assay (37). No such in vitro techniques are available for assay of ST, which is now usually tested for with the suckling mouse assay (12). These tests are generally accepted for determining the enterotoxigenicity of strains of *E. coli*. Their application to isolates of *Klebsiella pneumoniae* obtained from persons with acute infectious diarrhea has shown that some of these strains also yield a positive response in the CHO (16, 18) and other (11, 36) assay systems.

In vivo perfusion of various enterotoxin preparations of strains of *E. coli*, *Klebsiella*, and *Enterobacter cloacae* evokes net water secretion in the small intestines of experimental animals (3, 17, 20, 24, 33), and perfusion in rat jejuna of graded concentrations of ultrafiltrate fractions containing *E. coli* LT or ST toxins has been reported to provide an assay system with a sensitivity over a 10^6-fold range for the effect of these toxins on water transport (25). Strains of *E. coli*, *Klebsiella*, and *E. cloacae* isolated from persons with infectious diarrheal disease, tested by this technique, have been found to elaborate highly potent toxin materials, whereas those obtained from healthy, control sources either were inactive or produced only weakly active toxin material (20, 21).

This study was done to compare the results of assay for toxin activity by means of in vivo perfusion with results of several of the conven-

tional test systems, including the Y-1 adrenal and CHO cell assay for LT and the suckling mouse assay for ST. These tests were applied to 18 strains of *E. coli*, *Klebsiella*, or *E. cloacae* isolated from persons with infectious diarrheal disease and to a similar number of strains of the same species obtained from healthy, control sources.

MATERIALS AND METHODS

Strains examined. Eighteen of the 36 strains examined were provided by the Rochester laboratory and 18 by the Charlottesville laboratory. Strains were selected on the basis of their source, from healthy persons or those with an infectious diarrheal disease, irrespective of whether previous assays had shown a positive response or not. One-half of the strains had not been tested by perfusion at the time of selection. All strains were coded so that the other testing laboratories did not know their source until the study was over. Of the 18 strains from persons with diarrheal disease, 7 were isolated from the jejunum or feces of individuals with acute undifferentiated diarrhea in either Calcutta (13), Brazil (18), or Charlottesville (16), and 11 were cultured from the jejunum of persons with untreated tropical sprue in either Puerto Rico or Haiti (23). Six of the 18 strains obtained from control sources were cultured from the feces of healthy individuals living in a temperate zone, and 12 were isolated from the genitourinary tract. Biotypes were determined by the API 20E microtube profile system (Analytab Products, Inc., Plainview, N.Y.) (34).

Assay techniques. All 36 strains were negative for invasive properties when tested by the Sereny test (34) in Atlanta. Unconcentrated broth filtrates were tested by the CHO and suckling mouse assays in Charlottesville and by the Y-1 adrenal cell assay in Atlanta, using previously published techniques (6, 12, 15). Toxin preparations were tested by perfusion in Rochester. The procedures used in preparing the ultrafiltrate toxin preparations and in assaying them by in vivo marker perfusion in the rat jejunum have been described in detail (20, 24, 25). Briefly, preparations referred to as LT consisted of the material retained after whole-cell lysates, derived by sonic oscillation (Branson Sonic Power Co., Plainview, N.Y.) of harvested confluent surface growth on Trypticase soy agar, were passed through a PM-30 ultrafiltration membrane (Amicon Corp., Lexington, Mass.). The fractions referred to as ST consisted of the material retained after the acetone precipitate of an aerobic culture in Trypticase soy broth was passed sequentially through UM-10 and UM-05 ultrafiltration membranes.

Serial 10-fold dilutions of the ultrafiltrates, in balanced electrolyte solution that was kept isosmolar at 317 mosmol/kg with the plasma of this rat strain (24), were perfused for six 30-min test periods through the jejuna of anesthetized, tracheostomized Charles River Sprague-Dawley rats using a model 1201 Harvard peristaltic pump (Harvard Apparatus Co., Millis, Mass.). The net transport of water was calculated from changes in the concentration of polyethylene glycol 4000 by the usual marker technique formula (29). For reasons related to the prompt onset of action of ST versus the delayed onset of LT during perfusion, which we have discussed in detail previously (20, 25), the values used were for that 30-min perfusion period during which there was maximal secretion or minimal absorption. The presence of water secretion was interpreted to indicate an enterotoxin effect, and the value reported is the lowest concentration of toxin (in dry weight per milliliter) that induced secretion; this is referred to as the minimal effective concentration. On the basis of previous experience with this assay system (20, 21, 26), we have arbitrarily designated as positive those toxin fractions that evoked secretion at a perfusion concentration of 10 ng/ml or less and as negative those that either were inactive or produced secretion only at concentrations of greater than 10 ng/ml.

The failure to induce water secretion of similarly prepared ultrafiltrate preparations from control material such as uninoculated culture medium, and the heat lability characteristics of the ultrafiltrate toxin fractions of several of the strains used in this study, were described in earlier reports on perfusion studies (20, 25, 26).

RESULTS

Table 1 presents the results of tests by the conventional assays and by in vivo perfusion for each of the 18 strains isolated from persons with infectious diarrheal disease. Several strains lost their capacity to react positively in one or more of the conventional assay systems during the 1-year period of time during which these studies were being carried out. The value reported for this particular assay in these strains is the one obtained at the time of in vivo perfusion. *E. coli* strain H-10407 had become inactive in the suckling mouse assay prior to perfusion and is reported as negative. *Klebsiella* strain B 37-4 became inactive in the CHO assay, and strain TS 19-9 became inactive in both the CHO and suckling mouse assays subsequent to testing by perfusion; these strains are described as positive in these assays. No such loss of activity was noted by the perfusion assay when two of these strains (*E. coli* H-10407 and *Klebsiella* TS 9-19) were retested after storage for 6 months in Trypticase soy broth plus 15% glycerine at −60°C.

The LT ultrafiltrate preparations of six of the seven strains of *E. coli* isolated from diarrheal sources reacted positively in the perfusion assay; five of the six active strains also reacted positively in both the CHO and Y-1 adrenal cell assays (Fig. 1). The ST ultrafiltrate preparations of six of these strains were positive in the perfusion assay; two of the active strains were also positive in the suckling mouse assay. Both perfusion and conventional assays were negative in all seven strains obtained from control sources. Both ultrafiltrate preparations of two control strains and one ultrafiltrate fraction of two oth-

TABLE 1. *Assay results for strains isolated from diarrheal sources*

Strain[a]	Source[b]	Toxin	MEC[c] (ng/ml)	CHO[d] (%)	Y-1	SM[e]
E. coli						
334 (5044572)	AUD	LT	1	16	Pos	
		ST	0.1			0.1187
B 16-4 (5044572)	AUD	LT	1	15	Pos	
		ST	1			0.1258
B 21-4 (5044572)	AUD	LT	10	28	Pos	
		ST	10			0.0690
H-10407 (5044552)	AUD	LT	10	31	Pos	
		ST	10			0.0684
HAS 16F (5044552)	TS	LT	10	30	Pos	
		ST	10^3			0.0867
TS 5-16 (5044572)	TS	LT	NTG	2	Neg	
		ST	1			0.0713
HAS 7A (5044572)	TS	LT	1	8	Neg	
		ST	10			0.0747
Klebsiella						
B 37-4 (5215773)	AUD	LT	10	14	Neg	
		ST	100			0.0719
R-4 (1215773)	AUD	LT	10	2	Neg	
		ST	10			0.0756
MD-6 (5205773)	AUD	LT	10	3	Neg	
		ST	10			0.0634
TS 9-19 (5205773)	TS	LT	1	24	Neg	
		ST	0.1			0.0935
HAS 13J (5205773)	TS	LT	10	6	Neg	
		ST	10^5			0.0669
AT 1 (5205773)	TS	LT	100	6	Neg	
		ST	10			0.0745
TS 7-5 (5215773)	TS	LT	10^3	7	Neg	
		ST	10			0.0760
E. cloacae						
TS 3-11 (3305763)	TS	LT	10^3	6	Neg	
		ST	10			0.0801
TS 8-27 (3305573)	TS	LT	10	7	Neg	
		ST	1			0.0727
TS 13 (3305773)	TS	LT	10	9	Neg	
		ST	100			0.0801
TS 26 (3305773)	TS	LT	10	1	Neg	
		ST	100			0.0791

[a] Strain number. API profile biotype number is given in parentheses.
[b] AUD, Acute undifferentiated diarrhea; TS, tropical sprue.
[c] MEC signifies the minimum effective concentration in the rat perfusion assay. Values of 10 ng/ml or less are considered positive. NTG indicates absence of secretory activity.
[d] Values of >13.5% are regarded as positive in the CHO assay.
[e] SM, Suckling mouse assay. Values of >0.0875 are considered positive.

ers were completely inactive when perfused; ultrafiltrates of the other three strains induced water secretion only at concentrations of either 10 or 100 μg/ml.

The LT ultrafiltrate preparations of five of the seven *Klebsiella* strains isolated from persons with diarrhea were positive in the perfusion assay; two of the five active strains reacted positively in the CHO assay, but none of the seven was active in the Y-1 adrenal cell assay. The ST ultrafiltrate preparations of five of these strains were positive in the perfusion assay, but only one strain reacted positively in the suckling mouse assay. All seven strains from control sources were negative in both the conventional and perfusion assays. Both ultrafiltrate preparations of five of the control strains were completely inactive, and those of the other two strains evoked water secretion only at a high concentration.

The LT ultrafiltrate preparations of three, and the ST preparations of two, of the four strains

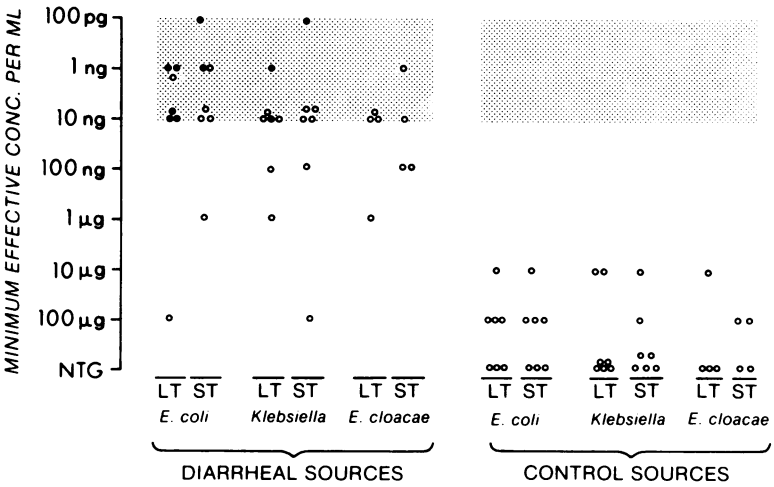

FIG. 1. *Results of in vivo perfusion of ultrafiltrate toxin preparations. The stippled area indicates the range of values regarded as indicative of significant enterotoxigenicity in this assay system. Conventional assay results: ●, positive; ○, negative.*

of *E. cloacae* obtained from diarrheal sources were positive in the perfusion assay. None of the strains reacted positively in any of the conventional assays. None of the four control strains reacted positively in the perfusion or conventional assays. Both ultrafiltrate preparations from one control strain and single preparations from three others were inactive when perfused; the other three preparations yielded secretion only at a high concentration.

To determine whether the failure of the conventional assays to detect LT in strains that reacted positively in the perfusion assay resulted from an insufficient amount of toxin material in the unconcentrated crude broth filtrates used in the conventional assays, additional in vitro assays were performed using the same concentrated LT ultrafiltrate material that was perfused. Three strains were examined, all of which had reacted positively for LT in the perfusion assay. The minimum amount of ultrafiltrate material necessary to induce secretion when added to the perfusates (the concentration per milliliter × 100 ml) was 0.1 µg in the case of *E. coli* strains 334 and HAS 7A and 1 µg for *Klebsiella* strain HAS 13J. Serial dilutions of the ultrafiltrate material of these strains containing 40, 4, and 0.4 µg were tested in the CHO and Y-1 adrenal cell assays. The unconcentrated broth filtrate of *E. coli* strain 334 was active in both in vitro assays, and both 40 and 4 µg, but not 0.4 µg, of the ultrafiltrate material also reacted positively in both of these test systems. The unconcentrated broth filtrates of both *E. coli* HAS 7A and *Klebsiella* HAS 13J were inactive in the in vitro assays, and ultrafiltrate preparations of both of these strains also failed to react even at the highest concentration tested.

DISCUSSION

The purpose of the present study was to determine whether strains of coliform bacteria which are found to produce potent toxin materials when tested by in vivo perfusion also stimulate conventional assay systems. Our results indicate that, among strains isolated from persons with infectious diarrheal disease, less than one-half of those that reacted positively in the perfusion assay for LT and/or ST yielded a positive response in the corresponding conventional assay for that particular toxin form. The correlation between the results of the perfusion and conventional assays was especially poor among strains of *Klebsiella* and *E. cloacae*. These observations are in accord with previous findings that strains of enteropathogenic serotypes of *E. coli* which are inactive in conventional assays produce potent toxin forms detectable only by in vivo perfusion (26), and with the findings reported by Nalin and his colleagues, who described strains of *E. coli* that were negative in the suckling mouse assay but had detectable ST activity when concentrated supernatants were perfused through canine jejuna (30).

The nature of the toxin materials present in concentrated ultrafiltrates that are responsible for water secretion is unclear. We have found that perfusion of a purified preparation of the polymyxin-release form of LT from *E. coli* strain H-10407 evokes water secretion in the same manner, in terms of onset of action and dosage required, as the LT ultrafiltrate preparation of

this strain (Klipstein and Engert, unpublished data). On this basis, it seems reasonable to regard the LT and ST materials present in ultrafiltrates from strains that are positive in the routine assays as conventional LT and ST. We have also used "LT" and "ST" to refer to the toxin materials present in ultrafiltrates of strains that were inactive in the routine assays, since they have the same range of apparent molecular weights, share heat-lability characteristics, and have the same prompt or delayed onset of action as purified preparations of these toxins and ultrafiltrate preparations derived from strains that are active in conventional assay systems. Nevertheless, it is quite apparent that a difference exists between the toxin materials elaborated by strains that are active in conventional assay systems and those that are not.

It is possible that the difference is a quantitative one related to methodology. Culture supernatants tested by conventional assays are unconcentrated, whereas those tested by perfusion are separated on the basis of molecular weight and concentrated by ultrafiltration. The limited information available suggests that this factor is not usually responsible for the differences noted. In the present study, LT assay, using CHO and Y-1 adrenal cells, of the concentrated ultrafiltrate material that was used for perfusion yielded a positive response only in the case of a strain that had been recognized as positive by assay of unconcentrated culture filtrates; ultrafiltrates from two strains that were negative by conventional testing failed to stimulate either in vitro assay even when added in amounts that were 40 times greater than that necessary to induce water secretion during perfusion. Nalin and his colleagues tested a 100-fold concentration of culture supernatants of strains of *E. coli* that were found to be positive for ST by perfusion and negative when unconcentrated culture filtrates were assayed in the suckling mouse; the concentrated material yielded a consistently positive response in the suckling mouse assay for only some of these strains.

These observations suggest that, although the different responses in the perfusion and conventional assay systems may be attributable to quantitative factors in some instances, in others this variation appears to be due to qualitative differences. It is uncertain whether the toxin materials that are detectable only by in vivo perfusion represent partially inactivated forms of the conventional toxins, which are no longer capable of stimulating the routine assay systems or totally dissimilar material. Studies concerned with the purification of conventional LT produced by enterotoxigenic strains of *E. coli* have shown that several heterogenous toxic products are produced which stimulate different assay systems (27). Previous analysis in this laboratory of toxin materials that have been defined as potent on the basis of perfusion failed to identify any difference between specific fractions, separated on the basis of molecular weight by sequential ultrafiltration, derived from strains of *E. coli* that were active or inactive in conventional assays (26).

For the past several years, investigations of the prevalence of enterotoxigenic forms of *E. coli* or other coliform species as pathogens of diarrheal disease have relied almost exclusively on the results of conventional assay systems. A negative result in these assays is construed as evidence that the strain under evaluation is not enterotoxigenic and hence not capable of inducing water secretion unless it is invasive. Recently accrued data, however, suggest that there are some strains of coliform bacteria that induce water secretion by noninvasive means and are not detected as enterotoxigenic by the conventional assays. (i) Strains of *E. coli* that can induce diarrheal disease in rabbits have been found to elaborate *Shigella dysenteriae*-like toxin rather than conventional forms of LT and ST toxin (31). (ii) Porcine strains of *E. coli* have been identified which produce a form of ST that is active in weaned pigs and rabbit ligated ileal loops but inactive in the suckling mouse assay (2). (iii) The observations in the present study and those by Nalin et al. (30) indicate that some strains of coliform bacteria isolated from persons with infectious diarrheal disorders produce toxins that cause water secretion but are not detected by routine conventional assays. Similarly, strains of enteropathogenic serotypes of *E. coli*, which were clearly incriminated on epidemiological grounds as the cause of nursery outbreaks of infantile diarrhea but which did not react in conventional assays for enterotoxins, were found to produce potent LT- and ST-like toxins capable of inducing water secretion during in vivo perfusion in the rat jejunum (26). The pathogenicity of several of these strains was confirmed by Levine and his associates, who demonstrated that their peroral administration to human volunteers produced diarrhea (28).

Several lines of evidence now suggest that *Klebsiella* may act as an enterotoxigenic pathogen responsible for diarrheal disease. (i) It has been found to represent the predominant bacterial species contaminating the gastrointestinal tract of some children (5, 19, 32) and adults (13) with acute diarrhea, and of some persons with tropical sprue (23). (ii) Some of these strains have been shown to yield positive results in the

CHO or other conventional assay systems (11, 16, 18, 36). (iii) *Klebsiella* serotype 19 has been incriminated on epidemiological grounds as the pathogen responsible for one nursery outbreak of acute diarrhea (32), and CHO-positive strains of *Klebsiella* have been incriminated in another nursery outbreak (16). (iv) Strains isolated from persons with diarrheal disease have been shown to elaborate toxin materials capable of inducing water secretion during in vivo perfusion (20, 21). (v) Intestinal monocontamination by a strain of *Klebsiella* (strain TS-9, serotype 19), defined as enterotoxigenic by both perfusion and conventional assays, was associated with intestinal water secretion in gnotobiotic rats, whereas monocontamination by a nontoxigenic strain did not affect intestinal transport (22). In the case of *E. coli*, virulence is the consequence of both toxigenicity and properties that promote adhesion to the mucosal surface, resulting in colonization of the small bowel (10). Certain strains of *Klebsiella* are found to have fimbriae that enhance their adhesive properties (8), but whether this factor is a significant aspect of the intestinal virulence of this organism is unknown. The effect of peroral administration of *Klebsiella* to normal volunteers has been evaluated in only one study. The administration of a single dose of a strain of serotype 19, which had been isolated during a nursery outbreak of acute diarrhea, failed to induce diarrhea in two volunteers (32). Bicarbonate or cimetidine were not administered concomitantly to counteract or ablate gastric acidity, nor was it ascertained whether these particular strains were enterotoxigenic. It would seem important, therefore, that this type of study be repeated using a strain with defined enterotoxigenic and adhesive properties.

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